J. Dairy Sci. 104:4206–4222
https://doi.org/10.3168/jds.2020-19746
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The effects of air stress during storage and low packing density on the fermentation and aerobic stability of corn silage inoculated with *Lactobacillus buchneri* 40788

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ABSTRACT

We determined if a microbial inoculant could improve the fermentation and aerobic stability of corn silage subjected to various challenges during storage that included an air stress challenge and low packing density. In Experiment 1, whole-plant corn was untreated (CTR) or treated (INO, *Lactobacillus buchneri* 40788 and *Pediococcus pentosaceus* 12455. Five individually replicated 7.5-L silos, at a density of 240 kg of dry matter (DM)/m^3, for each treatment were kept sealed (NAS) for 19 wk, air stressed early (ES, 3 h/wk for wk 1–9), or air stressed late during storage (LS, 3 h/wk for wk 10–19). Inoculation increased the number of agar-culturable lactic acid bacteria regardless of air stress status, but it did not affect the relative abundance of *Lactobacillus*. Early, but not late air stress, resulted in silages with a higher relative abundance of *Acetobacter* when compared with NAS. Silages treated with INO had greater concentrations of acetic acid than CTR. Numbers of yeasts were lowest for INO regardless of air stress and CTR-LS had the most yeasts among all treatments. Silages that were not air stressed had a higher relative abundance of *Candida tropicalis* than air stressed silages. *Monascus purpureus* was detected in ES and LS but not in NAS, and its relative abundance was numerically higher in CTR-ES than in INO-ES and statistically higher in CTR-LS compared with INO-LS. Early air stress numerically reduced aerobic stability compared with NAS, and there was a statistical tendency for lower stability in LS compared with NAS. Inoculation improved aerobic stability regardless of when the air stress occurred. In Experiment 2, corn silage was prepared with the same primary treatments of CTR and INO but was packed at a low (LD; 180 kg of DM/m^3) or a normal (ND; 240 kg of DM/m^3) density and sealed (NAS) or air stressed (AS; 24 h on d 28, 42, and 89) for 92 d of storage. The concentration of acetic acid was greater in INO compared with CTR and in AS compared with NAS. Numbers of yeasts were lower in NAS compared with AS regardless of inoculation and they were lower in INO-AS compared with CTR-AS. Treatment with INO improved aerobic stability but the improvement was better in NAS versus AS and better in ND versus LD. Overall, our experiments corroborate past findings showing that INO markedly improves the aerobic stability of corn silage but they are the first to show that improvement can be sustained even when the silage was exposed to regular air stresses and when packed at a low density.

Key words: aerobic stability, *Lactobacillus buchneri*, air stress, density

INTRODUCTION

The presence of air in a silage mass during ensiling, storage, or feed out is undesirable, as it allows for the growth of microorganisms that cause spoilage, resulting in a loss of DM, energy, and nutrients (Woolford, 1990). Feeding spoiled silage to ruminants can cause reductions in intake (Gerlach et al., 2013) and production (Whitlock et al., 2000). During early ensiling, aerobic microbes compete with lactic acid bacteria (**LAB**) for substrate. After forage has ensiled, infiltration of air into the silage mass during storage and feed out is known to stimulate lactate-assimilating yeasts and *Acetobacter*, which can initiate aerobic spoilage in silages (Pahlow et al., 2003). Thus, in most laboratory experiments, silos are quickly filled, densely packed, sealed immediately, and kept sealed during storage to minimize the exposure of forage and silage to air. However, in farm-scale silos, it is difficult to completely exclude air from the silage mass during silo filling and prevent its infiltration during storage (Borreani et al., 2018).

Received October 6, 2020.
Accepted November 30, 2020.
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For example, stressors such as low packing densities, compromises to the integrity of silo coverings, poorly secured plastic coverings, and leakage at sidewalls of bunker silos can result in silage being exposed to air even before being removed from the silo for feeding. Pitt (1986) concluded that even in typical farm silos, losses of DM due to infiltration of air were predicted to be 1 to 3% per month of storage. Ruppel et al. (1995) conducted a seminal study on silo density and showed that silage density was negatively correlated with DM losses during storage in farm-scale silos but a farm survey of 153 Californian dairies from the 2012 corn harvest season reported that 64.8% of the surveyed did not evaluate silo packing density (Heguy et al., 2016) suggesting that many farms may not be paying close attention to this crucial detail.

Lactobacillus buchneri has been added to forages at ensiling to improve aerobic stability (Muck, 1996; Ranjit and Kung, 2002) because it is able to convert moderate amounts of lactic acid primarily to acetic acid and 1,2 propanediol under anaerobic conditions (Oude Elferink et al., 2001) resulting in a suppression of yeasts that often initiate aerobic spoilage. Specifically, many studies have shown its effectiveness in corn silage (Ranjit and Kung, 2000; Reich and Kung, 2010), which is one of the primary silages commonly fed to lactating cows. However, only a few studies have shown the effectiveness of *L. buchneri* in corn silage under a variety of stressful conditions in the silo. Gallo et al. (2018) reported that the addition of a strain of *L. buchneri* improved aerobic stability of corn silage when ensiled at different densities, but the effect of packing density was minor in that study, probably because silos were well sealed during ensiling. Herrmann et al. (2015) reported that several inoculants containing various strains of *L. buchneri* were able to improve aerobic stability even when silages were air stressed during storage, but they did not vary density in their study. Neither of these studies compared challenging silages early versus late during storage or combined the stresses of low packing density and air stress during storage on silages treated with *L. buchneri.* Our hypothesis was that various stressors such as infiltration of air into the silo during storage and poor packing density, would detrimentally affect silage fermentation and aerobic stability and that use of a highly effective inoculant could moderate negative effects arising from those stressors. Thus, the objectives of our studies were to determine the effects of air stress during storage and varying packing densities with or without a microbial inoculant containing *L. buchneri* 40788 on the fermentation and aerobic stability of corn silage.

MATERIALS AND METHODS

Experiment 1

In the first year, whole-plant brown midrib corn (P1449AMX, DuPont Pioneer, Johnston, IA) was harvested using a pull-behind chopper (John Deere 3975, Moline, IL) with kernel processor (roller gap setting of 1.40 mm) at approximately 34% DM and chopped to a theoretical length of 19 mm. We applied treatments with a spray bottle and mixed into 5 individually replicated 25-kg piles of freshly chopped forage for each of the following primary treatments: no inoculant (200 mL of water; **CTR**) or treated with inoculant (**INO**; *Lactobacillus buchneri* 40788, final theoretical application rate of 400,000 cfu/g of fresh forage and *Pediococcus pentosaceus* 12455, 100,000 cfu/g of fresh forage; Lallemand Animal Nutrition, Milwaukee, WI, applied in 200 mL of water). For both primary treatments, 5 individually replicated silos were prepared from each pile for no air stress (**NAS**), early air stress (**ES**; subjected to air stress at wk 1–9 of ensiling) or late air stress (**LS**; subjected to air stress at wk 10–18 of ensiling). Forages were packed into 7.57-L food-grade pails (#20256, Encore Plastics Corp., Sandusky, OH) and sealed with plastic lids with O-ring seals (#200002, Encore Plastics Corp.). We packed silos with a gas-powered hydraulic press (a custom modified log splitter) to achieve a packing density of approximately 240 kg of DM/m^3. Silos subjected to air stress had 3 holes, each being 1.60 cm in diameter, plugged with butyl rubber stoppers and sealed with silicone glue. Two holes were located 5 cm above the bottom of the silo, 180° from each other, and the third hole was on the lid of the silo. All silo lids had a release valve fitted to rubber tubing leading to a water trap that allowed for the release of silage gas for the first week of fermentation but was permanently sealed thereafter. During air stress, we removed the rubber stoppers from the holes, allowing exposure to air for a unique 3-h period per week. After exposure to air, the stoppers were reinserted into their respective holes and sealed with silicone glue. The silos were stored at 22 ± 1°C and opened after 126 d of storage.

We determined the DM content of samples of freshly chopped whole-plant corn and of silages using a 100-g sample placed in a 60°C forced-air oven for 48 h, and the DM recovery was calculated based on the initial and final weights of the mass in the silo and the DM content of the forages and silages. Representative samples equal to 25.0 ± 0.1 g of fresh forage or silage were mixed with 225 mL of sterile quarter strength Ringer's solution (Oxoid BR0052G, Oxoid, Unipath,

Ltd., Basingstoke, UK) and homogenized for 1 min in a Proctor-Silex 57171 blender (Hamilton Beach/Proctor-Silex Inc., Washington, NC). The homogenate was used for pH measurement (pH/mV/Ion/°C/°F Ion700 Meter, Oakton, Vernon Hills, IL). Ten-milliliter portions of the homogenate were filtered through 4 layers of cheesecloth and a Whatman 54 filter paper (Whatman Ltd. Florham, NJ), acidified with 25 μL of a 50% (vol/vol) H_2SO_4 solution, and frozen (−18°C) before the analyses of the fermentation end products. Those extracts were analyzed for VFA via HPLC (RID-10A, Shimadzu Corporation, Nakagyo-ku, Kyoto, Japan) with sample preparation as per Muck and Dickerson (1988) and for NH_3-N and water-soluble carbohydrates by the colorimetric methods of Okuda et al. (1965) and Nelson (1944), respectively.

The following assays were performed by Cumberland Valley Analytical Services (Waynesboro, PA). A portion of the samples dried by forced-air oven as previously described was ground and passed through a 1-mm screen using a Udy Cyclone Mill (Udy Corp., Fort Collins, CO) and used for the analysis of NDF and ADF contents. The NDF was quantified as outlined by Van Soest et al. (1991), using a heat-stable amylase and sodium sulfite, and the ADF was quantified according to the procedures defined by Goering and Van Soest (1970), with the modification that the ADF residue was recovered on a 1.5-μm particle retention 7-cm Whatman glass fiber filter in a California Buchner Funnel instead of a Gooch crucible, to allow for better filtration. The analysis of NDF and ADF was not sequential, and the concentrations of NDF and ADF are reported on a DM (not ash-free) basis. The concentration of total N was determined by the combustion of the samples (Leco CNS 2000 Analyzer; Leco Corp., St. Joseph, MI) and the CP content was calculated by multiplying the total N content by 6.25. Soluble protein (**sol-N**, % of CP) was determined according to the procedure of Krishnamoorthy et al. (1982) on dried samples ground to pass through a 3-mm screen using a Wiley Mill (Thomas Scientific, Swedesboro, NJ). The starch content was determined and corrected for free glucose as per Hall (2009).

The same fresh water-extracts used to evaluate the pH were filtered through 4 layers of cheesecloth and used to determine the agar-culturable populations of LAB, yeasts, and molds. Numbers of LAB were determined by pour-plating 10-fold serial dilutions on de Man, Rogosa, and Sharpe agar (CM3651, Oxoid, Unipath, Basingstoke, UK). Agar plates were incubated anaerobically by being stored in containers that were placed in nylon-polyethylene bags (3.5-mm thickness, Doug Care Equipment Inc., Springville, CA), which were vacuumed to remove air and sealed with a PolyScience vacuum machine (distributed by Doug Care Equipment Inc., Springville, CA). An Anaero-Pack (10–01, Mitsubishi Gas Chemical Company Inc., New York, NY) for the generation of an anaerobic environment and an Anaerobic Indicator (BR0055B, ThermoScientific, Oxoid Ltd., Unipath) to ensure that anaerobic conditions were achieved were placed in each container. Plates were counted after 48 to 72 h of incubation at 35°C. Total numbers of yeasts and molds were determined by pour-plating 10-fold serial dilutions on malt extract agar (CM0059, Oxoid, Unipath) that was acidified with 0.5% (vol/vol) of 85% lactic acid after autoclaving. These plates were incubated aerobically for 48 h at 30°C before counting. Before the experiment, the microbial inoculant was plated in a 10-fold serial dilution series on de Man, Rogosa, and Sharpe agar and incubated anaerobically as previously described. Colony counts from these plates were used to formulate the amount of inoculant required to meet targeted application levels. A sample of the actual inoculant/water mix used on the day of treatment was also plated on de Man, Rogosa, and Sharpe agar and with the volume of liquid applied and the total weight of forage treated, was used to calculate the actual LAB application rate.

Samples of 4 replicates of fresh forage and silages were analyzed for the composition of bacterial and fungal communities by the sequencing of the V4-V5 region of the 16S rRNA and the internal transcribed spacer 1 (**ITS1**), respectively, using the Illumina MiSeq (Illumina, San Diego, CA) platform. Representative samples of 25.0 ± 0.1 g were mixed with 225 mL of autoclaved Ringer's solution (Oxoid BR0052G, Oxoid, Unipath) in a Colworth 400 stomacher (Seward, London, UK) for 2 min and then filtered through 4 layers of cheesecloth. Next, 2 mL of the homogenate were centrifuged at 21,130.2 × *g* for 3 min at room temperature (22 ± 1°C) in a Centrifuge 5424 R (Eppendorf AG, Hamburg, Germany). After centrifugation, the supernatant was discarded, and the pellet was resuspended by the addition of 100 μL of autoclaved Ringers solution. The samples were kept at −80°C until further analysis. DNA extraction, amplification, and Illumina MiSeq-based sequencing were performed by the Research and Testing Laboratory (Lubbock, TX). The DNA was extracted using the MoBio PowerMag Soil kit (MoBio Laboratories Inc., CA) according to the manufacturer's instructions. The primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 926R (5′-CCGTCAATTCMTTTRAGTTT-3′; Baker et al., 2003) were used to amplify the V4-V5 hypervariable regions of the 16S rRNA gene and the primers ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS2aR (5′-GCTGCGTTCTTCATCGATGC-3′; White et al., 1990; Gardes and Bruns, 1993) were used to amplify the ITS1.

Sequencing was done on an Illumina MiSeq platform employing the 2 × 250 base pair paired-end method. Sequences of the V4-V5 hypervariable regions of the 16S rRNA gene and of the ITS1 have been deposited in the NCBI Short Read Archive under the Bioproject ID PRJNA678991 and PRJNA679048, respectively. Data analysis was performed on Qiime 2 (Bolyen et al., 2019). Paired-end reads were trimmed from position 20 to 240, denoised, and merged, before being tabulated as operational taxonomic units using DADA2 (Callahan et al., 2016). Bacterial taxonomy was assigned with a naïve Bayes classifier trained under Qiime 2 2017.10.0 with the 99% identity sequence and classification files from Greengenes version 13.8 (DeSantis et al., 2006) and UNITE version 7.2 release 2017–10–10 (Nilsson et al., 2019), for bacteria and fungi, respectively. Species-level classifications with confidence <70% were discarded and replaced with classifications of decreasing specificity until a confidence of ≥70% was obtained. The outputs from Qiime 2 were processed in R version 3.6.2 (R Core Team, 2019). Operational taxonomic units classified as chloroplast or mitochondria were removed using the Phyloseq package (McMurdie and Holmes, 2013). Bray-Curtis dissimilarity was used to calculate the distance matrices to build principal coordinates analysis plots using the Phyloseq package (McMurdie and Holmes, 2013) in R (R Core Team, 2019). Statistical analysis of the distance matrices was performed by permutational multivariate ANOVA using the Adonis function on Vegan (Oksanen et al., 2013). A matrix of Pearson correlation coefficients was built using the corrplot package (Wei and Simko, 2017) in R (R Core Team, 2019).

The aerobic stability of fermented corn silage was determined on 2 kg of a representative sample placed in clean 7.57 L buckets. The temperature of the silage mass was recorded every 15 min using thermocouple wires, placed in the geometric center of silage mass, attached to a data logger (DT85 Series 3, Thermo Fisher Scientific Australia Pty. Ltd., Scoresby, VIC, Australia). Aerobic stability was defined as the length of time (h) before the temperature of the silage mass rose 2°C above the baseline temperature of each silo after exposure to air at an ambient temperature of 22 ± 1°C. Silages were exposed to air for a minimum of 10 d.

Numbers of yeasts, molds, and LAB were $\log_{10}$-transformed before statistical analysis and presented as $\log_{10}$ values. The data for Experiment 1 were analyzed as a completely randomized design in a 2 × 3 factorial arrangement of treatments with factors including the effect of additive (**ADI**) and air stress (**AST**) and their interaction. Data were analyzed using the Fit Model procedure in JMP (SAS Institute Inc., Cary, NC). Data for the effect of ADI and AST were tested using Tukey's test (Snedecor and Cochran, 1980) only if there were no significant interactions ($P > 0.05$) and if significance was detected for the specific effect ($P \leq 0.05$). When a significant interaction was detected ($P \leq 0.05$), means were tested using Tukey's test (Snedecor and Cochran, 1980).

Experiment 2

In a second year, we used whole-plant brown midrib corn (F2F817 116RM, Mycogen Seeds, Indianapolis, IN) that was harvested using a pull-behind chopper (John Deere 3975) with kernel processor (roller gap setting of 1.40 mm) at about 37% DM and chopped to a theoretical length of 19 mm. Replicated piles of CTR and INO were prepared as described in Experiment 1 and ensiled at a low density (**LD**; 180 kg of DM/m^3) or a normal density (**ND**; 240 kg of DM/m^3). For each treatment and density combination, 5 individually replicated silos were stored without air stress (NAS) or with air stress (AS) for 24 h on d 28, 42, and 89 of storage. Air stress was a modification of the evaluation scheme for silage additives (DLG, 2013). All silos were stored at 22 ± 1°C and opened after 91 d of ensiling. All methods were as previously described for Experiment 1 with the exception that there was no analysis for microbial communities. We analyzed the data for Experiment 2 similarly to that of Experiment 1 except that there was a 2 × 2 × 3 factorial arrangement of treatments with factors including the effect of ADI, the effect of AST, and the effect of packing density (**DN**) and their interactions.

RESULTS

Experiment 1

The chemical and microbial analysis of the freshly chopped corn plants before treatment are shown in Table 1. The DM of the fresh corn was 34.24%, and the pH was 5.64. The populations of LAB, yeasts, and molds were 6.53, 5.87, and 4.42 $\log_{10}$ cfu/g of fresh forage, respectively. Based on counts of the applied inoculant used on the day of ensiling, we determined that inoculation supplied an actual count of 548,070 cfu of LAB/g of fresh forage, which is in agreement with the targeted application rate of 500,000 cfu/g of fresh forage.

The chemical composition of silages after 126 d of ensiling is shown in Table 2. We found no effect of inoculation or air stress on the DM, concentration of NH_3-N, and DM recovery. Crude protein and sol-N were greatest ($P = 0.03$ and $P < 0.01$, respectively) in NAS silages (9.51% DM and 58.28% of CP, respec-

Table 1. The chemical (% DM basis unless stated otherwise) and microbial composition (log_{10} cfu/g fresh weight basis) of freshly chopped corn plants before treatment in Experiment 1

Item	Value ± SD
DM, %	34.24 ± 0.97
pH	5.64 ± 0.01
CP	8.64 ± 0.07
Sol-N % of CP	32.52 ± 0.82
NH_3-N	0.04 ± 0.01
ADF	23.12 ± 0.59
NDF	40.68 ± 0.88
Starch	30.14 ± 1.01
Water-soluble carbohydrates	11.47 ± 0.49
Lactic acid bacteria	6.53 ± 0.03
Yeasts	5.87 ± 0.04
Molds	4.42 ± 0.07

tively) compared with silages exposed to late air stress (9.21% DM and 53.07% of CP, respectively). Additionally, INO silages (55.70%) had greater ($P = 0.02$) sol-N concentration on a CP basis than CTR silages (53.77%). The concentration of ADF was higher ($P = 0.01$) in silages exposed to early air stress (25.19% DM) compared with no air stress (23.09% DM) and late air stress silages (22.96% DM). The concentration of NDF was higher ($P = 0.04$) in ES than in NAS (40.28 vs. 37.66% DM). Inoculated silages (0.23%) had lower ($P < 0.01$) concentrations of water-soluble carbohydrates on a DM basis than CTR silages (0.87%). Early air stress silages had the lowest ($P = 0.04$) concentrations of starch (28.20% DM) compared with the other types of air-stressed silages (31.94% DM).

The pH, fermentation end products, and microbial populations of silages ensiled for 126 d are shown in Table 3. We found an interaction ($P < 0.01$) between air stress and the use of the additive for pH because pH was higher in INO than CTR silages for NAS ($P = 0.01$, 3.78 vs. 3.73) and LS ($P < 0.01$, 3.78 vs. 3.73) but it was similar in ES silages ($P = 1.00$, 3.77 and 3.76). The concentration of lactic acid was not affected by inoculation ($P = 0.64$), but there was a trend ($P = 0.06$) for the lactic acid content to be higher in NAS than in LS (4.80 vs. 4.08% DM). Silage air stressed early had a higher ($P = 0.02$) concentration of acetic acid than LS silages (2.13 vs. 1.52% DM). Treatment with INO resulted in a higher ($P < 0.01$, 2.28% DM) concentration of acetic acid compared with CTR (1.22% DM). Inoculation resulted in a higher concentration of 1,2 propanediol compared with uninoculated silages under

Table 2. The DM, chemical composition (% DM basis unless stated otherwise), and DM recovery of corn silage, treated with an inoculant and subjected to no air stress or air stress early or late during storage, ensiled for 126 d, Experiment 1

Item[1]	DM, %	CP	Sol-N,[2] % of CP	NH_3-N	ADF	NDF	WSC[3]	Starch	DMR[4]
NAS									
CTR	31.61	9.56	58.34	0.12	23.58	38.36	0.80	30.16	87.23
INO	32.14	9.46	58.22	0.13	22.60	36.96	0.27	33.72	88.75
ES									
CTR	31.98	9.34	51.92	0.11	25.90	40.60	0.91	26.86	88.30
INO	31.33	9.60	53.78	0.13	24.48	39.96	0.18	29.54	87.45
LS									
CTR	32.29	9.22	51.04	0.13	22.64	37.84	0.90	32.02	89.20
INO	32.45	9.20	55.10	0.13	23.28	37.80	0.24	31.86	89.23
Air stress means									
NAS	31.87	9.51^{a}	58.28^{a}	0.12	23.09^{b}	37.66^{b}	0.53	31.94^{a}	87.99
ES	31.66	9.47^{ab}	52.85^{b}	0.12	25.19^{a}	40.28^{a}	0.53	28.20^{b}	87.88
LS	32.37	9.21^{b}	53.07^{b}	0.13	22.96^{b}	37.82^{ab}	0.57	31.94^{a}	89.22
Additive means									
CTR	31.96	9.37	53.77^{b}	0.13	24.04	38.93	0.87^{a}	29.68	88.24
INO	31.97	9.42	55.70^{a}	0.13	23.45	38.24	0.23^{b}	31.71	88.48
SEM	0.33	0.11	0.91	0.01	0.64	0.99	0.07	1.46	0.97
Effects and interactions (P-value)									
AST	0.10	0.03	<0.01	0.37	<0.01	0.02	0.84	0.02	0.33
ADI	0.95	0.61	0.02	0.56	0.27	0.40	<0.01	0.10	0.77
AST × ADI	0.20	0.26	0.09	0.35	0.25	0.79	0.36	0.43	0.48

[a,b]Means in columns within a category with unlike superscripts differ ($P < 0.05$).

[1]NAS = no air stress during storage; CTR = untreated, control; INO = treated with a theoretical application rate of 400,000 cfu of *Lactobacillus buchneri* 40788/g of fresh forage and 100,000 cfu of *Pediococcus pentosaceus* 12455/g of fresh forage (Lallemand Animal Nutrition, Milwaukee, WI); ES = exposure to air for 3 h/wk for wk 1–9 of ensiling; LS = exposure to air for 3 h/wk for wk 10–18 of ensiling; AST = effect of air stress; ADI = effect of additive.

[2]Soluble protein.

[3]Water-soluble carbohydrates.

[4]DM recovery.

all storage conditions but the effect was not as great in ES ($P = 0.01$, 0.41 vs. 0.03% DM) and LS ($P < 0.01$, 0.50 vs. 0.04% DM) as it was in NAS ($P < 0.01$, 0.93 vs. 0.07% DM). There were no differences in the concentration of propionic acid among silages (average of 0.44% DM; data not shown). The concentration of ethanol tended to be increased by inoculation ($P = 0.06$, 2.66 vs. 2.40% DM) and was affected by air stress ($P = 0.04$). The concentration of ethanol tended to be lower in ES ($P = 0.06$, 2.39 vs. 2.77% DM) and LS ($P = 0.09$, 2.42 vs. 2.77% DM) than in NAS. Numbers of LAB were always greater in INO compared with CTR, but the effect was greater under NAS ($P < 0.01$, 8.13 vs. 6.23 $\log_{10}$ cfu/g) and LS ($P < 0.01$, 8.55 vs. 5.36 $\log_{10}$ cfu/g) than ES ($P = 0.01$, 8.60 vs. 7.28 $\log_{10}$ cfu/g). Numbers of yeasts were always lower in INO than CTR but the effect was greater in LS ($P < 0.01$, < 1.00 vs. 4.29 $\log_{10}$ cfu/g) than NAS ($P = 0.01$, 1.10 vs. 2.50 $\log_{10}$ cfu/g) or ES ($P = 0.01$, 1.44 vs. 2.79 $\log_{10}$ cfu/g). Molds were unaffected by air stress ($P = 0.47$), but they were higher ($P = 0.04$) in CTR compared with INO (1.92 vs. 1.34 $\log_{10}$ cfu/g).

The principal coordinates analysis plots of the bacterial and fungal communities in the fresh forage and silages are shown in Figure 1. For the bacterial and fungal analysis, a significant ($P < 0.01$) permutational multivariate ANOVA (Adonis) test proved that the different treatments did not have the same centroid, indicating that the bacterial and fungal community composition were not similar among all treatments. The permutation test for homogeneity of multivariate dispersion (betadisper) test was nonsignificant ($P = 0.24$) for the bacteria data set, showing that the result of the Adonis test was not caused by sample group dispersion, but it was significant ($P < 0.01$) for the fungi data set, indicating that the result of the Adonis test could have been caused by sample group dispersion. Figures 1A and 1B show that the fresh forage samples clustered separated from the silage samples, which indicates that the ensiling process affected the bacterial and fungal community composition, respectively. Figure 1A also shows that samples from CTR-NAS and CTR-LS formed a cluster separated from the samples of inoculated silages, and Figure 1B shows that samples from CTR-LS formed a unique cluster that was separated from other silage samples.

There were 11 bacterial families with a relative abundance (**RA**) higher than 1% of the total bacterial community in untreated freshly chopped whole-plant corn (Figure 2A): *Enterobacteriaceae* (20.36%), *Xan-*

Table 3. The pH, fermentation end products (% DM), and microbial populations ($\log_{10}$ cfu/g wet weight basis) of corn silage, treated with an inoculant and subjected to no air stress or to air stress early or late during storage, ensiled for 126 d, Experiment 1

Item[1]	pH	Lactic acid	Acetic acid	1,2 PD[2]	Ethanol	LAB[3]	Yeasts	Molds
NAS								
CTR	3.73^{bc}	4.69	0.96	0.07^{c}	2.65	6.23^{c}	2.50^{bc}	2.15
INO	3.78^{a}	4.90	2.23	0.93^{a}	2.89	8.13^{ab}	1.10^{d}	1.40
ES								
CTR	3.77^{ab}	4.30	1.77	0.03^{c}	2.16	7.28^{b}	2.79^{b}	1.56
INO	3.76^{ab}	4.05	2.49	0.41^{b}	2.62	8.60^{a}	1.44^{cd}	1.24
LS								
CTR	3.73^{c}	4.23	0.92	0.04^{c}	2.39	5.36^{c}	4.29^{a}	2.05
INO	3.78^{a}	3.92	2.12	0.50^{b}	2.46	8.55^{a}	<1.00^{d}	1.39
Air stress means								
NAS	3.76	4.80	1.60^{ab}	0.50	2.77	7.18	1.80	1.78
ES	3.76	4.18	2.13^{a}	0.22	2.39	7.94	2.11	1.40
LS	3.75	4.07	1.52^{b}	0.27	2.42	6.96	2.65	1.72
Additive means								
CTR	3.74	4.41	1.22^{b}	0.04	2.40	6.29	3.19	1.92^{a}
INO	3.77	4.29	2.28^{a}	0.61	2.66	8.42	1.18	1.34^{b}
SEM	0.01	0.30	0.21	0.07	0.16	0.23	0.25	0.32
Effects and interactions (P-value)								
AST	<0.01	0.05	<0.01	<0.01	0.04	<0.01	<0.01	0.47
ADI	0.25	0.64	0.02	<0.01	0.06	<0.01	<0.01	0.04
AST × ADI	<0.01	0.65	0.40	<0.01	0.50	<0.01	<0.01	0.79

$^{a-d}$Means in columns within a category with unlike superscript differ ($P < 0.05$).

[1]NAS = no air stress during storage; CTR = untreated, control; INO = treated with a theoretical application rate of 400,000 cfu of *Lactobacillus buchneri* 40788/g of fresh forage and 100,000 cfu of *Pediococcus pentosaceus* 12455/g of fresh forage (Lallemand Animal Nutrition, Milwaukee, WI); ES = exposure to air for 3 h/wk for wk 1–9 of ensiling; LS = exposure to air for 3 h/wk for wk 10–18 of ensiling; AST = effect of air stress; ADI = effect of additive.

[2]1,2-propanediol.

[3]Lactic acid bacteria.

thomonadaceae (16.26%), *Sphingomonadaceae* (7.34%), *Sphingobacteriaceae* (7.07%), *Alcaligenaceae* (2.95%), *Pseudomonadaceae* (1.47%), *Burkholderiaceae* (1.40%), *Rhizobiaceae* (1.28%), *Comamonadaceae* (1.20%), *Methylobacteriaceae* (1.15%), and *Brucellaceae* (1.05%); and 7 fungal families (Figure 2B): *Hypocreales_fam_Incertae_sedis* (27.71%), *Saccharomycetales_fam_Incertae_sedis* (14.05%), *Nectriaceae* (11.02%), *Didymellaceae* (8.90%), *Pleosporaceae,* (3.27%), *Cladosporiaceae* (2.34%), and *Sporidiobolaceae* (1.48%). After ensiling, the bacterial genera (Figure 2C) with RA higher than 1% were *Lactobacillus* (93.03%) and *Acetobacter* (1.43%) and the fungal genera (Figure 2D) were *Candida* (41.13%), *Monascus* (15.87%), *Kazachstania* (4.61%), *Mycosphaerella* (4.30%), *Rhodosporidiobolus* (4.26%), *Fusarium* (2.97%), *Meyerozyma* (1.78%), *Occultifur* (1.35%), *Scedosporium* (1.03%), and *Cladosporium* (1.02%). We identified 7 fungal species that were present in silages at a RA of more than 1%: *Candida tropicalis* (33.65%), *Monascus purpureus* (15.87%), *Candida quercitrusa* (7.48%), *Kazachstania humilis* (4.61%), *Mycosphaerella tassiana* (4.30%), *Meyerozyma guilliermondii* (1.79), and *Scedosporium boydii* (1.03%) (data not shown). From those species, only *C. tropicalis* and *M. purpureus* were affected by inoculation or air stress. The only species observed in the genus *Monascus* was the *M. purpureus* and *C. tropicalis* comprised 81.82% of the genus *Candida*.

Table 4 shows the RA of the bacteria genera that were present in the silages at a RA of more than 1% and that were affected by inoculation or air stress. We found that the RA of *Lactobacillus* was not affected by inoculation ($P = 0.64$) or air stress ($P = 0.13$). The RA of *Acetobacter* was not affected by inoculation and it was not detected in NAS, but ES had a higher RA of *Acetobacter* (4.06%) than LS ($P < 0.01$, 0.41%).

Candida, *Monascus*, and *Occultifur* were the only fungi genera with a RA greater than 1% that were affected by inoculation or air stress (Figure 2D). A greater RA of *Candida* was observed in inoculated silages than in CTR ($P = 0.01$, 51.08 vs. 29.77%), and in NAS than in ES ($P < 0.05$, 60.51 vs. 33.55%) and LS silages ($P < 0.01$, 60.51 vs. 25.21%). There was an interaction between inoculation and air stress ($P < 0.01$) for the RA of *Monascus*, which was undetectable in CTR-NAS, INO-NAS, and INO-ES, and was greater in CTR-LS than in CTR-ES ($P < 0.01$, 79.63 vs. 14.97%) and INO-LS ($P < 0.01$, 79.63 vs. 0.41%). The RA of *Occultifur* was greater in NAS than in ES silages ($P = 0.04$, 2.71 vs. 0.29%). Table 4 shows the RA of the fungi species that were present in the silages at a RA of more than 1% and that were affected by inoculation or air stress. Air-stressing silages early ($P = 0.04$, 25.40%) and late ($P = 0.02$, 22.14%) resulted in a lower RA of *C. tropicalis* when compared with NAS (51.84%). Inoculated silages tended to have a higher ($P = 0.08$) RA of *C. topicalis* than CTR (40.41 vs. 25.85%). There was an interaction ($P < 0.01$) between inoculation and air stress for the RA of *M. purpureus.* The RA of *M. purpureus* was higher ($P < 0.01$) in CTR-LS (79.63%) than in other treatments (average of 3.08%). The organism *M. purpureus* was not detected in NAS silages but its RA numerically increased in CTR-ES (14.97%) and increased markedly ($P < 0.01$) in CTR-LS (79.63%)

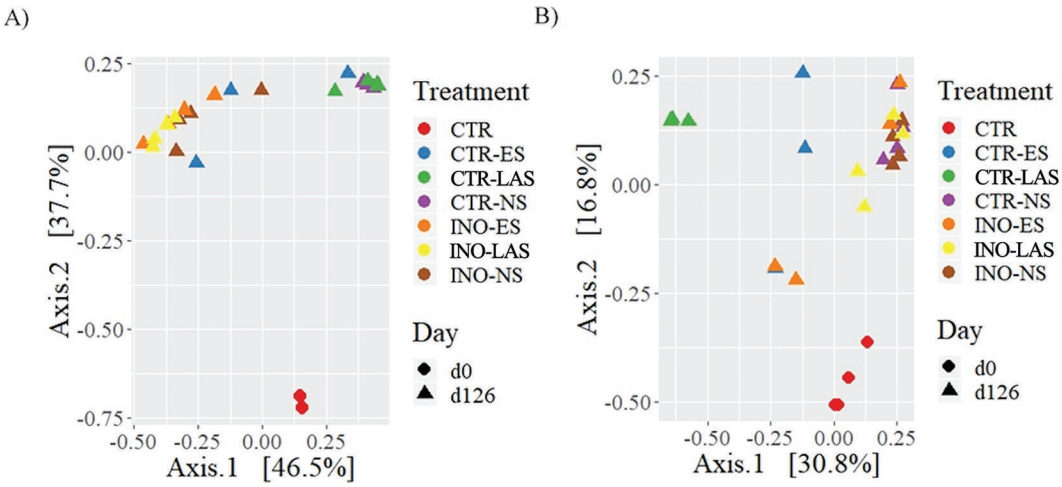

Figure 1. Principal coordinates analysis plots with Bray-Curtis dissimilarity of the composition of the (A) bacterial and (B) fungal communities of untreated (CTR) fresh forage (d 0), and silages (d 126) untreated (CTR) or treated with an inoculant (INO, 400,000 cfu of *Lactobacillus buchneri* 40788/g fresh forage and 100,000 cfu of *Pediococcus pentosaceus* 12455/g of fresh forage, Lallemand Animal Nutrition, Milwaukee, WI) and not air stressed (NAS) or air stressed early (ES, 3 h/wk for wk 1 to 9) or late (LS, 3 h/wk for wk 10 to19) during storage, ensiled for 126. Experiment 1.

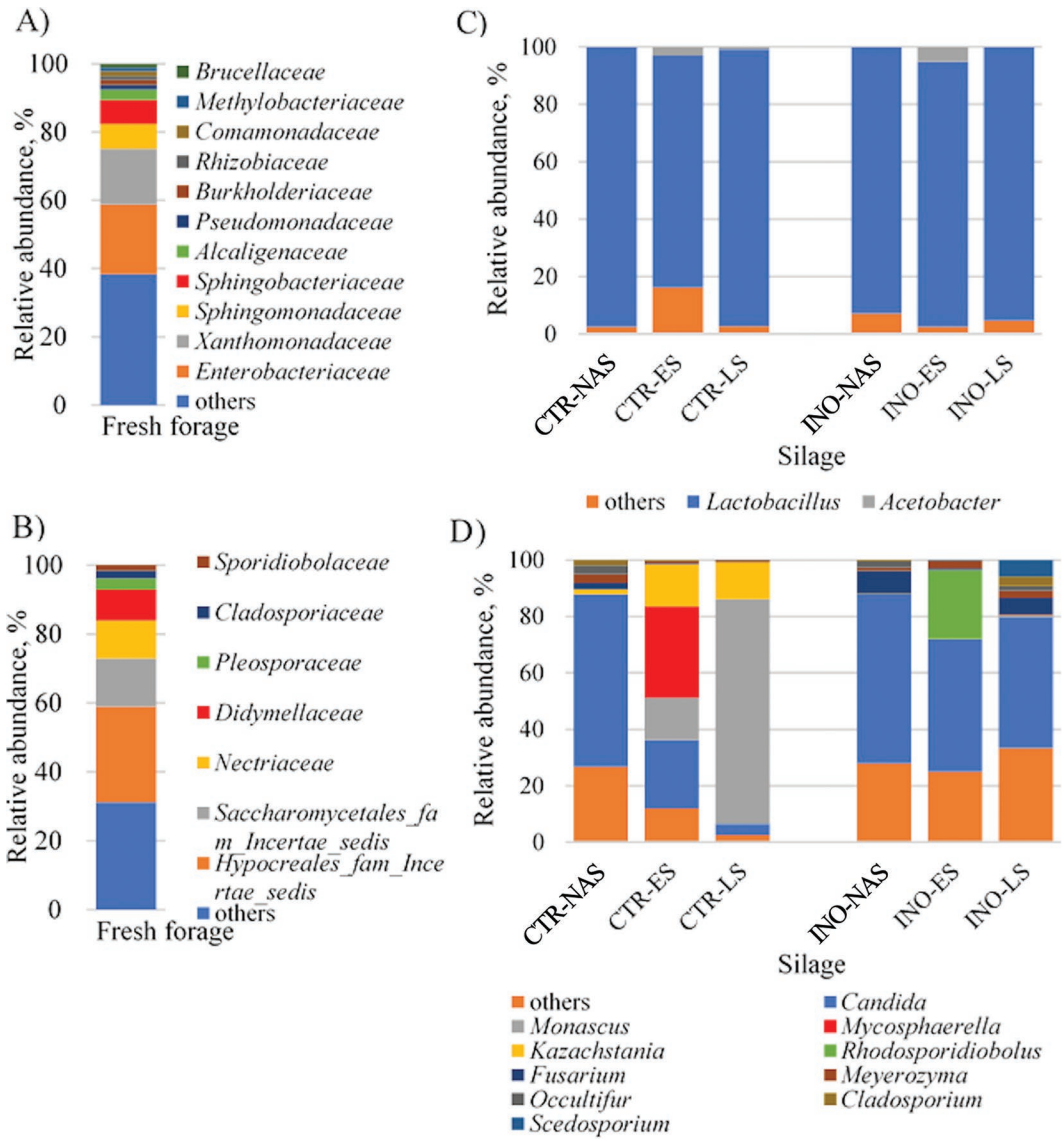

Figure 2. Average relative abundance (%) of 4 replicates per treatment of the (A) bacterial and (B) fungal families in freshly chopped corn plants before treatment and of the (C) bacterial and (D) fungal genera in corn silages, untreated (CTR) or treated with an inoculant (INO, 400,000 cfu of *Lactobacillus buchneri* 40788/g fresh forage and 100,000 cfu of *Pediococcus pentosaceus* 12455/g of fresh forage, Lallemand Animal Nutrition, Milwaukee, WI) and not air stressed (NAS) or air stressed early (ES, 3 h/wk for wk 1 to 9) or late (LS, 3 h/wk for wk 10 to 19) during storage, ensiled for 126 d. Families and genera with a relative abundance lower than 1% were grouped under "others." Experiment 1.

but not in INO-ES (not detected) or INO-LS (0.41%). Although large numerical differences were present for the RA of the *Kazachstania* genus, the variability was great and thus statistical differences were not detected among treatments. The RA of the genus *Occultifur* was higher ($P = 0.04$) in NAS versus ES but it was not different ($P = 0.11$) from LS.

There was a tendency ($P = 0.09$) for aerobic stability to be lower in LS (140 h) than NAS (227 h; Figure 3). Regardless of air stress, INO silages were more stable ($P < 0.01$, 279 h) compared with CTR silages (76 h).

Experiment 2

The chemical and microbial composition of the freshly chopped corn plants before treatment is shown in Table 5. The DM of the fresh corn was 36.68% and the pH was 6.25. Fresh forage contained 7.66% DM CP, 0.02% DM NH_3-N, and its soluble protein content was 27.08% of CP. The concentrations of ADF, NDF, and starch were 21.28, 37.48, and 39.22% DM, respectively. Fresh forage contained 6.62 $\log_{10}$ cfu of LAB, 4.41 $\log_{10}$ cfu of yeasts, and 3.46 $\log_{10}$ cfu of molds per gram of

Table 4. Relative abundance (%) of the bacterial genera and fungal species in corn silages, treated with an inoculant and subjected to no air stress or to air stress early or late during storage, ensiled for 126 d, as analyzed by the sequencing of the V4–V5 region of the 16S rRNA, for bacteria, and the internal transcribed spacer 1, for fungi, using the Illumina MiSeq platform[1]

	Bacteria relative abundance		Fungi relative abundance	
Item[2]	*Lactobacillus*	*Acetobacter*	*Candida tropicalis*	*Monascus purpureus*
NAS				
CTR	97.44	0.00	53.38	0.00[b]
INO	92.80	0.00	50.31	0.00[b]
ES				
CTR	80.89	2.94	21.05	14.97[b]
INO	92.25	5.19	29.75	0.00[b]
LS				
CTR	96.52	0.76	3.11	79.63[a]
INO	95.26	0.06	41.17	0.41[b]
Air stress means				
NAS	95.12	0.00[b]	51.84[a]	0.00
ES	86.57	4.06[a]	25.40[b]	7.48
LS	95.89	0.41[b]	22.14[b]	40.02
Additive means				
CTR	91.62	1.23	25.85	31.53
INO	93.43	1.75	40.41	0.14
SEM	5.30	0.93	10.84	5.52
Effects and interactions (P-value)				
AST	0.13	<0.01	0.01	<0.01
ADI	0.64	0.46	0.08	<0.01
AST × ADI	0.25	0.22	0.11	<0.01

[a,b]Means in columns within a category with unlike superscripts differ $P < 0.05$.

[1]The table only shows genera that had a relative abundance of more than 1% and were affected by inoculation or air stress. Experiment 2. NAS = no air stress during storage; CTR = untreated, control; INO = treated with a theoretical application rate of 400,000 cfu of *Lactobacillus buchneri* 40788/g of fresh forage and 100,000 cfu of *Pediococcus pentosaceus* 12455/g of fresh forage (Lallemand Animal Nutrition, Milwaukee, WI); ES = exposure to air for 3 h/wk for wk 1–9 of ensiling; LS = exposure to air for 3 h/wk for wk 10–18 of ensiling; AST = effect of air stress; ADI = effect of additive.

fresh forage weight. Based on counts of the applied inoculant used on the day of ensiling, we determined that inoculation supplied an actual count of 548,070 cfu of LAB/g of fresh forage (data not shown), which is in agreement with the targeted application rate of 500,000 cfu/g of fresh forage.

Table 6 shows the DM, chemical composition, and DM recovery of corn silage after 92 d of ensiling. We found that air stress, treatment with the inoculant, and varying silage density did not affect these measurements. The exception that there was an interaction ($P = 0.01$) between the effects of additive and air stress for sol-N because it was higher in NAS than AS for both CTR and INO, but the difference was higher for INO ($P < 0.01$, 52.70 vs. 46.14% of CP) than CTR ($P < 0.01$, 50.74 vs. 47.41% of CP). There was a tendency ($P = 0.06$) for DMR to be higher in INO (93.15%) compared with CTR (91.21%).

Table 7 shows the pH, fermentation end products, and microbial populations of corn silages after 92 d of fermentation. We detected that air stress caused the final silage pH to be higher ($P < 0.01$) compared with NAS (3.69 vs. 3.61) and INO silages also had higher ($P < 0.01$) pH than CTR (3.71 vs. 3.59). Packing density did not affect ($P = 0.11$) silage pH. The concentration of lactic acid was not affected by inoculation ($P = 0.38$) or air stress ($P = 0.11$), but it was higher ($P < 0.01$) in ND (5.53% DM) versus LD (4.37% DM). Air stress resulted in silages with higher ($P = 0.02$) acetic acid concentration when compared with NAS (2.13 vs. 1.67% DM) and INO (2.39% DM) was higher ($P < 0.01$) in acetic acid than CTR (1.41% DM). Packing density also did not affect ($P = 0.26$) the concentration of acetic acid. There was an additive and air stress interaction ($P = 0.01$) for the concentration of 1,2 propanediol because it was not detectable in any of the CTR silages, but it was present in all INO silages being higher ($P < 0.01$) in INO silage not exposed to air (0.95% DM) compared with INO silages exposed to air (0.78% DM). We did not detect propionic in the silages. Air stress resulted in silages with lower ($P = 0.01$) concentrations of ethanol (1.14% DM) compared with silages without air stress (1.28% DM). The ethanol concentration was higher ($P < 0.01$) in ND (1.28%) compared with LD (1.14%). Inoculation did not affect ($P = 0.86$) the concentration of ethanol. Numbers of LAB were higher ($P < 0.01$) in INO than CTR for both LD and ND but the difference was higher in ND (8.66 vs. 6.60 $\log_{10}$ cfu/g) than in LD (8.68 vs. 7.14 $\log_{10}$ cfu/g). An interaction was found between additive and air stress for the numbers

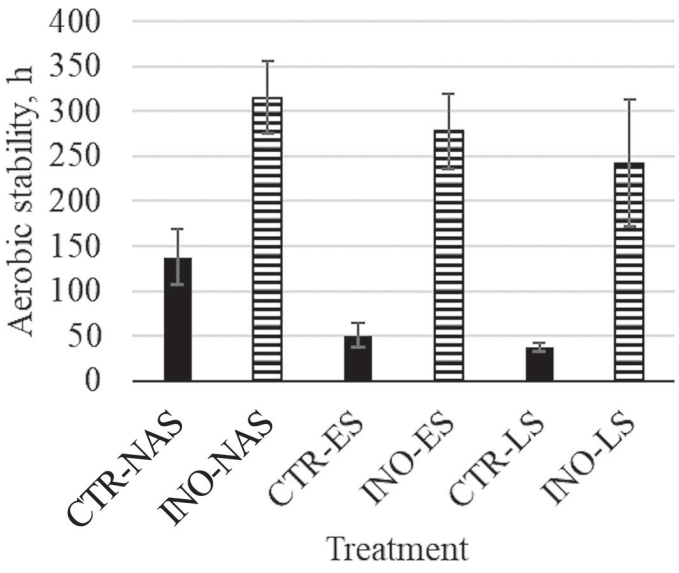

Figure 3. The aerobic stability (h) of corn silage in Experiment 1 ensiled for 126 d. Aerobic stability was defined as a 2°C increase in temperature over the established baseline temperature. SEM = 43. INO (279 h) > CTR (76 h), $P < 0.01$. NAS (227 h) > LS (140 h), $P < 0.09$. Corn silage that was either untreated (CTR) or treated with an inoculant (INO, 400,000 cfu of *Lactobacillus buchneri* 40788/g fresh forage and 100,000 cfu of *Pediococcus pentosaceus* 12455/g of fresh forage, Lallemand Animal Nutrition, Milwaukee, WI) and not air stressed (NAS) or air stressed early (ES, 3 h/wk for wk 1 to 9) or late (LS, 3 h/wk for wk 10 to 19) during storage.

of LAB ($P = 0.05$), yeasts ($P = 0.02$), and molds ($P = 0.01$). Numbers of LAB were higher ($P < 0.01$) in INO than CTR for both NAS and AS but the difference was higher in AS (8.77 vs. 6.74 $\log_{10}$ cfu/g) than in NAS (8.57 vs. 7.00 $\log_{10}$ cfu/g). The CTR silages had more ($P < 0.01$) yeasts than INO silages when air stressed (5.63 vs. 4.28 $\log_{10}$ cfu/g) but the same ($P = 0.85$) numbers of yeasts as INO when not air stressed (2.24 vs. < 2.00 $\log_{10}$ cfu/g). The numbers of molds were not affected ($P = 0.65$) by inoculation (2.16 to 2.84 $\log_{10}$ cfu/g) in silages not subjected to air stress, but they were the highest ($P = 0.02$) in uninoculated silage with air stress (4.89 $\log_{10}$ cfu/g) compared with inoculated silages (3.77 $\log_{10}$ cfu/g). We also found a tendency ($P = 0.06$) for LD silages to have greater numbers of molds than ND silages.

Table 5. Chemical (% DM basis unless stated otherwise) and microbial analysis ($\log_{10}$ cfu/g fresh weight basis) of freshly chopped corn plants before treatment, Experiment 2

Item	Value ± SD
DM, %	36.68 ± 0.35
pH	6.25 ± 0.02
CP	7.66 ± 0.10
Soluble protein, % of CP	27.08 ± 0.91
NH_3-N	0.02 ± 0.01
ADF	21.28 ± 0.54
NDF	37.48 ± 0.93
Starch	39.22 ± 1.93
Water-soluble carbohydrates	2.02 ± 0.20
Lactic acid bacteria	6.62 ± 0.03
Yeasts	4.41 ± 0.24
Molds	3.46 ± 0.09

The aerobic stability of silages after 92 d of ensiling is shown in Figure 4. There was an interaction ($P = 0.04$) between DN and AS because air stress caused a larger decrease in stability in low-density silage ($P < 0.01$, 198 to 49 h) than in normal-density silage ($P < 0.01$, 184 to 77 h). An additive and air stress interaction ($P < 0.01$) was also found because overall aerobic stability was lowest for untreated with air stress (28 h), intermediate for inoculated silage with air stress (97 h) and untreated silage without air stressed (119 h), but highest for inoculated silage without air stress (263 h). There was also an interaction ($P < 0.01$) between density and air stress for aerobic stability because aerobic stability was improved more by inoculation in normal density ($P < 0.01$, 62–199 h) than low-density silage ($P < 0.01$, 86–161 h).

DISCUSSION

Most laboratory studies ensile forages under ideal conditions to minimize stresses that might negatively affect the ensiling process. However, silages made on farms are subject to many challenges that can affect silage fermentation. In Europe, silage additives must be authorized before their sales in the marketplace (Pauly and Wyss, 2018). The German DLG system (DLG, 2013) requires inoculants to be tested in silages that are air stressed during storage to make a claim for improved aerobic stability and to obtain the DLG quality label. We previously pointed out that no one laboratory method would cover the different types of air stress that farm silages could encounter (Kung et al., 2018). Therefore, in the current studies, we chose to evaluate different types of stresses on the fermentation and aerobic stability of laboratory-scale silos. In Experiment 1, we evaluated the ability of an inoculant containing *L. buchneri* 40788 to improve aerobic stability when challenged with air stress either early versus late during storage, which might occur if damage to the plastic sheeting that covers bunker and pile silos or punctures in plastic used in bag and bale silages occur at different times of storage. A second objective with the early air stress challenge was to mimic a condition in which the problem with air infiltration is corrected, for example, by closing the holes in bag silos weeks before the silo is opened at feed out, and compare it to the late air stress challenge, in which the silo is opened before the correction of the problem with air infiltration. In Experiment

2, we combined 2 stressors, low packing density coupled that with air stress. Our objective when combining air stress challenge with low packing density was to mimic some of the conditions of farm-scale silos, such as the lower density and greater air exposure of the top layer compared with the innermost parts of the silo, or when the management conditions are not ideal, for example, when the forage is too dry and difficult to pack, facilitating air infiltration. In that experiment, we used a slightly modified version of the DLG air stress protocol (DLG, 2013) because, in their protocol, 24-h air stresses are conducted at 28 and 42 d of storage followed by silo opening and tests for aerobic stability at 49 d. However, it is well known that *L. buchneri* is a relatively slow-growing organism in silage and its full effects on improving aerobic stability have usually required ensiling periods of longer than 40 to 60 d (Kleinschmit and Kung, 2006; Schmidt et al., 2008, 2009). Therefore, we stored our silage for a longer period and added an extra day of air stress before opening silos. Although several published studies have air-stressed silos during storage, they have not always compared this to ideal conditions without air stress. Thus, we felt that it was important to compare results under both ideal and stressed conditions in our current experiments.

In both experiments, we found that air stress generally resulted in uninoculated silages with a higher pH, which was accompanied by lower concentrations of lactic acid. This was not surprising as air allows for the growth of lactate-assimilating yeasts (Pahlow et al., 2003; Santos et al., 2017) and can alter the use of sugars by some LAB (Condon, 1987). Similar results to air stress have been reported in high moisture corn (Pauly and Hjelm, 2015) and corn silage (Weiss et al., 2016). We found that air stress decreased the concentration of ethanol in both experiments, which could have been caused by the activity of acetic acid bacteria, which are obligate aerobic bacteria that can metabolize ethanol

Table 6. The DM (%) and chemical composition (% DM basis unless stated otherwise) of corn silage, treated with an inoculant, packed at different densities, and subjected to no air stress or air stress during storage, ensiled for 92 d, Experiment 2

Treatment[1]	DM	CP	Sol-N,[2] % of CP	NH_3-N	ADF	NDF	Starch	DMR[3]
NAS								
ND								
CTR	35.57	8.08	51.60	0.11	23.62	39.20	32.86	91.46
INO	35.91	8.32	51.82	0.11	24.50	40.80	32.60	93.17
LD								
CTR	35.82	8.12	49.88	0.12	23.06	38.26	35.48	92.08
INO	35.71	8.08	53.58	0.12	25.68	42.26	30.58	92.56
AS								
ND								
CTR	35.79	8.14	47.68	0.12	26.92	43.56	28.24	91.72
INO	36.19	7.86	46.36	0.12	25.32	41.32	30.64	93.46
LD								
CTR	34.93	8.24	47.14	0.13	24.34	40.52	31.90	89.58
INO	36.09	8.00	45.92	0.12	24.28	42.06	32.94	93.42
NAS								
CTR	35.70	8.10	50.74[a]	0.12	23.34	38.73	34.17	91.77
INO	35.81	8.20	52.70[a]	0.12	25.09	41.53	31.59	92.87
AS								
CTR	35.36	8.19	47.41[b]	0.12	25.63	42.04	30.70	90.65
INO	36.14	7.93	46.14[b]	0.12	24.80	41.69	31.79	93.44
SEM	0.51	0.13	0.84	0.01	0.98	1.48	1.96	1.37
Effects and interactions (P-value)								
AST	0.99	0.32	<0.01	0.22	0.16	0.11	0.17	0.78
DN	0.53	0.91	0.70	0.07	0.29	0.67	0.25	0.58
ADI	0.22	0.37	0.57	0.80	0.51	0.25	0.76	0.06
AST × DN	0.49	0.22	0.67	0.50	0.14	0.51	0.34	0.58
AST × ADI	0.36	0.06	0.01	0.60	0.07	0.14	0.13	0.39
DN × ADI	0.84	0.50	0.14	0.88	0.25	0.15	0.29	0.82
AST × DN × ADI	0.41	0.37	0.17	0.38	0.94	0.74	0.56	0.40

[a,b]Means in columns with unlike superscripts differ ($P < 0.05$).

[1]NAS = no air stress during storage; ND = packed at a normal density (240 kg DM/m^3); LD = packed at a low density (176 kg DM/m^3); CTR = untreated, control; INO = treated with a theoretical application rate of 400,000 cfu of *Lactobacillus buchneri* 40788/g of fresh forage and 100,000 cfu of *Pediococcus pentosaceus* 12455/g of fresh forage (Lallemand Animal Nutrition, Milwaukee, WI); AS = exposure to air for 24h on 28, 42, and 90 d of ensiling; AST =effect of air stress; DN = Effect of packing density; ADI = effect of additive.

[2]Soluble protein.

[3]Dry matter recovery.

Table 7. The pH, fermentation end products (% DM), and microbial populations (log_{10} cfu/g of fresh forage weight) of corn silage, treated with an inoculant, packed at different densities and subjected to air stress during storage, ensiled for 92 d, Experiment 2

Treatment[1]	pH	Lactic acid	Acetic acid	1,2 PD[2]	Ethanol	LAB[3]	Yeasts	Molds
NAS								
CTR	3.55	5.29	1.33	0.00^{c}	1.28	7.00^{b}	2.24^{c}	2.16^{c}
INO	3.66	5.08	2.00	0.95^{a}	1.28	8.57^{a}	<2.00^{c}	2.84^{bc}
AS								
CTR	3.63	4.86	1.48	0.00^{c}	1.15	6.74^{b}	5.63^{a}	4.89^{a}
INO	3.75	4.57	2.78	0.78^{b}	1.14	8.77^{a}	4.28^{b}	3.77^{b}
ND								
CTR	3.61	5.43	1.52	0.00	1.28	6.60^{c}	3.94	3.33
INO	3.72	5.62	2.50	0.91	1.28	8.66^{a}	3.17	2.83
LD								
CTR	3.58	4.72	1.30	0.00	1.15	7.14^{b}	3.93	3.73
INO	3.69	4.02	2.86	0.83	1.13	8.68^{a}	3.12	3.77
Air stress means								
NAS	3.61^{b}	5.18	1.67^{b}	0.48	1.28^{a}	7.78	2.12	2.50
AS	3.69^{a}	4.71	2.13^{a}	0.39	1.14^{b}	7.54	4.96	4.33
Density means								
ND	3.66	5.53^{a}	2.01	0.45	1.28^{a}	7.63	3.55	3.08
LD	3.64	4.37^{b}	1.79	0.41	1.14^{b}	7.91	3.52	3.75
Additive means								
CTR	3.59^{b}	5.08	1.41^{b}	<0.01	1.21	6.87	3.93	3.53
INO	3.71^{a}	4.82	2.39^{a}	0.87	1.21	8.67	3.14	3.30
SEM	0.02	0.41	0.27	0.04	0.07	0.15	0.34	0.81
Effects and interactions (P-value)								
AST	<0.01	0.11	0.02	0.01	0.01	0.78	<0.01	<0.01
DN	0.11	<0.01	0.26	0.16	<0.01	0.02	0.89	0.06
ADI	<0.01	0.38	<0.01	<0.01	0.86	<0.01	<0.01	0.50
AST × DN	0.17	0.78	0.60	0.06	0.77	0.29	0.34	0.50
AST × ADI	0.76	0.89	0.11	0.01	0.88	0.05	0.02	0.01
DN × ADI	0.93	0.14	0.98	0.16	0.81	0.02	0.92	0.44
AST × DN × ADI	0.45	0.37	0.89	0.06	0.54	0.27	0.23	0.15

[a–c]Means in columns with unlike superscripts differ ($P < 0.05$).

[1]NAS = no air stress during storage; CTR = untreated, control; INO = treated with a theoretical application rate of 400,000 cfu of *Lactobacillus buchneri* 40788/g of fresh forage and 100,000 cfu of *Pediococcus pentosaceus* 12455/g of fresh forage (Lallemand Animal Nutrition, Milwaukee, WI); AS = exposure to air for 24h on 28, 42, and 90 d of ensiling; ND = packed at a normal density (240 kg DM/m^3); LD = packed at a low density (176 kg DM/m^3); AST = effect of air stress; DN = effect of packing density; ADI = effect of additive.

[2]1,2-Propanediol.

[3]LAB = lactic acid bacteria.

to acetic acid (Spoelstra et al., 1988). In contrast, the concentration of acetic acid was affected differently in our experiments. In Experiment 1, both early and late air stress silages had concentrations of acetic acid similar to the ones of no air stress silages, but in Experiment 2, the acetic acid concentration was higher in air-stressed silage. In this case, air may have allowed obligate aerobic bacteria such as *Acetobacter* or facultative anaerobic organisms such as *Enterobacteria*, which produce acetic acid, to survive (Pahlow et al., 2003). Air stress also had some different effects on numbers of agar-culturable yeasts between experiments. It clearly increased the numbers of yeasts in Experiment 2, but in our first experiment, late, but not early air stress, increased the numbers of yeasts. The exact reasons for differing responses to air stress between studies are unknown but may be related to the different air stress regimens that were used. Few prolonged bursts of air stress, as used in Experiment 2, might have allowed for more development of yeasts compared with the shorter, although more frequent, air stresses in Experiment 1. These findings support those of Knicky et al. (2016) who stated that more frequent continuous aeration could be a more sensitive protocol to evaluate aerobic stability than longer limited exposures. Data from our first experiment also suggests that the timing of air stress may be important, and it appears silages are able to recover from air stress if it occurs early during storage rather than closer to silo opening. Late air-stressed CTR silage had the lowest LAB numbers after ensiling, while simultaneously having the highest number of yeasts. Silage exposed to late air stress also had the lowest CP concentration present after fermentation. These findings emphasize the need for consistent monitoring of silages throughout storage so that breaks in silo integrity that might allow for infiltration

of air can be quickly fixed. Additionally, we found the development of *Acetobacter* in air-stressed silages, especially in silages air-stressed early. *Acetobacter* is a genus of acetic acid bacteria that is commonly found in corn silage and can initiate the aerobic deterioration process, usually simultaneously with yeasts (Spoelstra et al., 1988). Because *Acetobacter* are aerobic bacteria, their development is usually detected in farm silos but not in laboratory-scale silos that are customarily well sealed (Guan et al., 2018). Muck et al. (1992) observed in laboratory conditions that when corn silage was stored under an atmosphere containing 100% CO_2 the numbers of acetic acid bacteria dropped below the detectable range of 2 $\log_{10}$ cfu/g of silage within one moth of ensiling. In farm-scale silos, Dolci et al. (2011), when comparing silo covering films, observed that after the silos were opened, *Acetobacter* was first detected in corn silages covered with polyethylene film than in silages covered with oxygen barrier film (2 vs. 7 d after silo opening). Da Silva et al. (2020), found that the RA of *Acetobacter* tended to be higher in corn silage ensiled in laboratory silos that were subjected to an air stress challenge than in silos that were completely sealed. Similarly, we observed at silo opening that *Acetobacter* was not detected in non-air-stressed silages but was only found in air-stressed silages, especially in silages subjected to air stress early during ensiling. Such findings indicate that methods of air-stressing laboratory silos, such as the ones applied by da Silva et al. (2020) or in the present study, can be used to artificially produce silages that contain acetic acid bacteria, enabling studies that attempt to investigate their mode of action or their control. The higher RA of *Acetobacter* observed in silages air stressed early compared with silages air stressed late can be explained by the fact that the inhibition of microorganisms by the low pH, fermentation end products, and anaerobic conditions of the silo is dependent on the length of exposure. Therefore, it was expected that the longer the *Acetobacter* population was exposed to the unfavorable silo conditions the more it would decrease. With that, when the silos were air stressed early, the initial *Acetobacter* population in the silage was larger and developed more when exposed to oxygen than when the silos were air stressed late and had an initial *Acetobacter* population too modest to respond to the favorable aerobic environment. It is also important to mention that the silos that were air stressed early remained completely sealed during the second half of the ensiling period, and despite the lack of air infiltration for 9 wk, *Acetobacter* was still detected at the end of the ensiling period. Therefore, further investigation on the effect of early exposure to air on farm-scale silos is important to analyze if, for example, rapid filling and sealing reduce the numbers of acetic acid bacteria of the final silage.

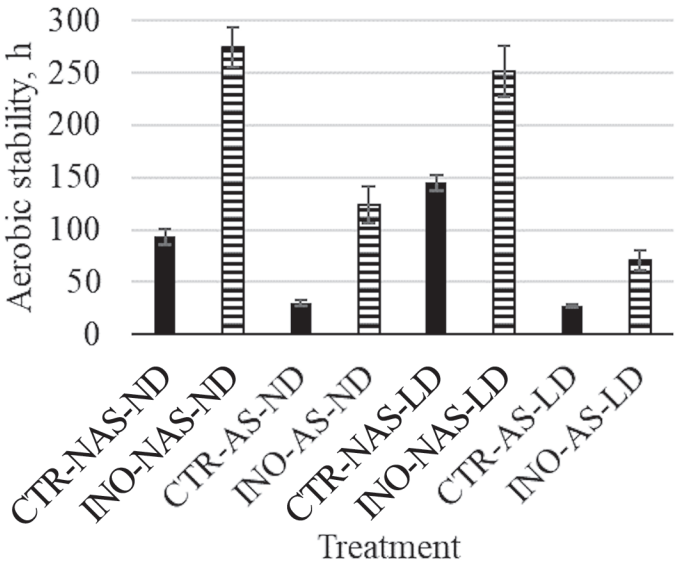

Figure 4. The aerobic stability (h) of corn silage in Experiment 2 ensiled for 92 d. Aerobic stability was defined as a 2°C increase in temperature over the established baseline temperature. There were interactions between packing density and inoculation ($P < 0.01$), air stress and packing density ($P = 0.04$), and inoculation and air stress ($P < 0.01$). SEM = 15. Corn silage that was either untreated (CTR) or treated with an inoculant (INO, 400,000 cfu of *Lactobacillus buchneri* 40788/g fresh forage and 100,000 cfu of *Pediococcus pentosaceus* 12455/g of fresh forage, Lallemand Animal Nutrition, Milwaukee, WI), packed to normal density (240 kg DM/m^3, ND) or low density (180 kg DM/m^3, LD), and not air stressed (NAS) or air stressed (AS, 24 h on 28, 42, and 89 d of ensiling).

Poor packing densities have clearly been shown to be undesirable in farm-scale silos (Ruppel et al., 1995). In contrast, its effects have been more variable under laboratory conditions. In corn silage, Velho et al. (2007) reported that silage packed at a high density conserved more fermentable sugars and had reduced proteolysis when compared with loosely packed silage. Sucu et al. (2016) similarly reported that loosely packed corn silage had higher concentrations of NH_3-N and a higher concentration of acetic acid than tight-packed silage. In our second experiment, a low packing density resulted in less lactic acid and ethanol than in a tighter pack and it tended to increase the numbers of molds in silage. However, Gallo et al. (2018) also reported only small differences in corn silage in fermentation profiles and yeasts and molds in poorly versus tightly packed laboratory silos. Varying effects of density on silage in laboratory silos could be due to differences in the original amount of air trapped in the silo mass at sealing and the integrity of the silos during storage. In fact, in the study of McEniry et al. (2007), although poor compaction alone had little effects on the fermentation of grass silages when coupled with air stress, it

did result in a greater loss of DM, more bacilli, and lower fermentation end products. Similarly, density interacted with air stress on its effect on aerobic stability in our second experiment because stability decreased more when low-density silos were air stressed compared with high-density silos that were air stressed.

Gallo et al. (2018) reported that the addition of a strain of *L. buchneri* improved aerobic stability of corn silage when ensiled at different densities, but the effect of packing density was minor in that study, probably because silos were well sealed during ensiling. In contrast, Sucu et al. (2016) reported that in corn silage, tightly packed silage had lower concentrations of acetic acid, NH_3-N, and lower DM loss than loosely packed silage. Herrmann et al. (2015) reported that several inoculants containing various strains of *L. buchneri* were able to improve aerobic stability even when air stress during storage, but they did not vary density in their study.

We found that inoculation had generally no effects on the nutrient compositions of silages, which has been reported before (Tabacco et al., 2011; Queiroz et al., 2012). However, it was clear that the added LAB from inoculation dominated the overall fermentation process, as these silages had markedly higher numbers of agar-culturable LAB. The main bacteria families found in fresh forage from Experiment 1 were *Enterobacteriaceae*, *Xanthomonadaceae*, *Sphingomonadaceae*, *Sphingobacteriaceae*, *Alcaligenaceae*, and *Pseudomonadaceae*, which is in agreement with the findings of Drouin et al. (2019) that reported *Pseudomonadales*, *Xanthomonadales*, *Enterobacteriales*, *Sphingobacteriales*, and *Flavobacteriales* as the main orders found in the fresh whole-corn plant analyzed. After ensiling the genus *Lactobacillus* dominated the fermentation in both uninoculated and inoculated silages regardless of air stress status, but in silages exposed to air stress during early ensiling, we observed a numerical reduction on the RA of *Lactobacillus* and the development of *Acetobacter*. Also, we detected the classical effects of treating forage with *L. buchneri* (Oude Elferink et al., 2001). Inoculation resulted in silages with a higher pH, which can be explained by these silages having numerically lower concentrations of lactic acid when compared with untreated silages. Lack of statistical differences in lactic acid between inoculated and untreated silages maybe a result of increased lactic acid production from the added *P. pentosaceus*. Inoculated silages also had higher concentrations of acetic acid and 1,2 propanediol regardless of being air stressed or not. However, air stress decreased the concentrations of lactic acid and 1,2-propanediol in inoculated silages even though it did not reduce the total numbers of LAB or the RA of *Lactobacillus*. Because there were no differences in the concentrations of propionic acid among treatments, a conversion of the 1,2 propanediol to propionic acid was unlikely. Therefore, a possible explanation to these findings is that even though the inoculant dominated the fermentation its heterofermentative pathway activity was compromised by the air stress.

Because acetic acid is highly antifungal (Moon, 1983), it followed that inoculation also reduced the numbers of agar-culturable yeasts and this was true even when silages were challenged with air stress at different times of storage. In the present work, the 2 main fungal genera found in the silages were *Candida* and *Monascus.* Similarly, Keshri et al. (2018) also found that *Candida* and *Monascus* were the main fungi in corn silage ensiled for 90 d, comprising 74 and 26% of the total fungal population, respectively. Molds from the genus *Monascus* are common in poorly managed corn silages and undesirable especially because those fungi can produce mycotoxins (Schneweis et al., 2001; Driehuis et al., 2018). Molds native to the plant, such as *M. purpureus,* can survive and develop in silages that are exposed to air and have a high pH due to prior deterioration by lactate-assimilating yeasts. Jiang et al. (2019) observed that *Candida* and *Pichia* were associated with the aerobic deterioration of a TMR silage containing wheat straw, and that *M. purpureus* was not present in a high abundance during the early stages of aerobic exposure but that its RA increased after 72 h of aerobic exposure. Therefore, the dominance of the fungal community of silages by molds rather than yeasts suggests that the silage is less stable and is undergoing an advanced stage of deterioration. Air stressing the silages early or late during storage resulted in lower RA abundance of *C. tropicalis* compared with silages that were not air stressed, indicating that air stressed silages were in a more advanced stage of deterioration than silages that were not air stressed. The same was true for uninoculated silages, which tended to have a lower RA of *C. tropicalis* than inoculated silages. *M. purpureus* comprised less than 0.5% of the RA of inoculated silages but in uninoculated silage air stressed early it reached 15% and in uninoculated silage air stressed late 80% of the total fungal population, indicating a more advanced degree of deterioration in those silages. Therefore, both the lack of air stress in the silo and inoculation maintained the fungal population more stable with a high RA of the yeast *C. tropicalis* and a low RA of the mold *M. purpureus*. Similarly, da Silva et al. (2020) also observed that the RA of *C. tropicalis* was lower in corn silages subjected to an air stress challenge than in silages that were not air stressed, but in this study instead of the fungal population of air-stressed si-

lages to shift toward the development of molds, another species of yeast, the *Pichia kudriavzevii*, overcame the *C. tropicalis* population.

The effect of INO on silage fermentation was even more dramatic than the effect of air stress. Regardless of air stress, treatment with INO overcame the negative effects of the stress that were seen in their CTR counterparts. The LAB populations were highest for all INO silages and yeasts were lowest compared with CTR silages, regardless of air stress. This microbiological data are supported by the fermentation profile of the INO silages. The INO silages had greater acetate and 1,2 propanediol than CTR for each air stress type. This is likely because *L. buchneri* degrades lactic acid to acetic acid, 1,2 propanediol, and ethanol under anaerobic conditions (Oude Elferink et al., 2001).

Knicky and Spörndly (2015) showed that a chemical additive was effective at improving aerobic stability under conditions of low density and air stress. In the present study, although inoculation with *L. buchneri* increased aerobic stability in low and normal density silos, the effect was less robust when density was low. Inoculation improved aerobic stability in air-stressed silos, but this effect was even greater in silos that were not air stressed.

CONCLUSIONS

To the best of our knowledge, these are the first experiments evaluating the effects of an inoculant containing *L. buchneri* 40788 on the fermentation and aerobic stability of corn silage that was stressed early versus late during storage and ensiled with different degrees of packing density with or without air stress during storage. They corroborate past findings showing that *L. buchneri* 40788 markedly improves the aerobic stability of corn silage, but they are the first to show that improvement can be sustained even when the silage was exposed to various stressors during storage. Use of *L. buchneri* 40788 in either of these air stress situations improved the aerobic stability of corn silage although the overall effect was dampened by air stress. We also found that negative effects of low packing density are exacerbated when coupled with air stress thus, combinations of stressful conditions during ensiling can be additive. Air stress, especially during early ensiling, allowed the survival of *Acetobacter*. We also found that air-stressing uninoculated silages during late ensiling decreased the abundance of the yeast *C. tropicalis* and caused the mold *M. purpureous* to dominate the fungal population, but inoculation with *L. buchneri* 40788 prevented such shift from a population dominated by yeasts to one dominated by molds. Our data suggests that air stress near silo opening rather than early in storage is more detrimental in negatively affecting aerobic stability. Thus, we highly encouraged producers to regularly monitor the integrity of their silos especially when they are close to opening the silos and follow all guidelines suggested to ensure a high-quality silage fermentation.

ACKNOWLEDGMENTS

These experiments were partially funded by Lallemand Animal Nutrition (Milwaukee, WI). The authors thank the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior–Brasil (CAPES) - Finance Code 001 for the scholarship [grant number 99999.013556/2013-04] and the farm crew at the University of Delaware (Newark, DE) for planting and harvesting of corn. The authors have not stated any conflicts of interest.

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